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Differential Expression of Immune Response Genes in Steller Sea Lions *(Eumetopias jubatus):* An Indicator of Ecosystem Health?

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Abstract: Characterization of the polygenic and polymorphic features of the Steller sea lion major histocompatibility complex (MHC) provides an ideal window for evaluating immunologic vigor of the population and identifying emergence of new genotypes that reflect ecosystem pressures. MHC genotyping can be used to measure the potential immunologic vigor of a population. However, since ecosystem-induced changes to MHC genotype can be slow to emerge, measurement of differential expression of these genes can potentially provide real-time evidence of immunologic perturbations. MHC DRB genes were cloned and sequenced using peripheral blood mononuclear leukocytes derived from 10 Steller sea lions from Southeast Alaska, Prince William Sound, and the Aleutian Islands. Nine unique DRB gene sequences were represented in each of 10 animals. MHC DRB gene expression was measured in a subset of six sea lions. Although DRB in genomic DNA was identical in all individuals, relative levels of expressed DRB mRNA was highly variable. Selective suppression of MHC DRB genes could be indicative of geographically disparate environmental pressures, thereby serving as an immediate and sensitive indicator of population and ecosystem health.

Key words: Steller sea lion, ecosystem health

The polygenic and polymorphic attributes of the major histocompatibility complex (MHC) are proposed to be central to maintaining the immunologic health and vigor of a species. MHC genes encode a set of transmembrane proteins critical to the generation of immune responses (Paul, 1999; Klein and Sato, 2000a,b). The variety of MHC-encoded proteins in an individual ultimately determines the repertoire of foreign peptides to which that animal is capable of responding and, at the population level, reflects the historic influence of pathogen pressures and breeding biology (Zinkernagel, 1979; Reizis et al., 1998). This MHC genetic diversity plays an important role in a host's ability to accommodate rapidly evolving infectious agents that periodically afflict natural populations (Yuhki and O'Brien, 1990). To date, MHC diversity has been delineated using

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genomic analysis. Such MHC genotyping has facilitated identification of individuals and/or populations with low levels of immunogenetic heterogeneity and attempted to associate this with both past pathogenic and environmental influences (Hedrick, 1994; Hughes and Hughes, 1995) and increased susceptibility to novel environmental insults.

Examination of the MHC provides an ideal window for evaluating the immunologic vigor of populations. However, the environment to which the Steller sea lions are currently exposed is changing relative to that in which the MHC evolved. Expression of these MHC genes is similarly influenced by environmental change and has the added benefit of reflecting real-time insults. Extensive literature has established relationships between physiologic insults (including environmental contaminants/chemicals, viruses, and nutritional stress) and reduced MHC class II expression (Snyder and Unanue, 1982; Hughes et al., 1996; Dong et al., 1997; Venkatraman and Pendergast, 2002; Schwab et al., 2005). Thus, extension of the genotyping approach, to include quantitative measurement of transcription of individual MHC genes, should provide a timely and relevant measure of altered host immune potential and environmental stress. In light of this concept, we developed a quantitative PCR system to examine MHC class II DRB gene expression in Alaskan Steller sea lions (SSL; *Eumetopias jubatus*) from two genetically distinct populations, the threatened Eastern and endangered Western stocks.

Ten SSLs ranging in age from 2 months to 2 years old were sampled from Prince William Sound (PWS), Southeast Alaska (SE), and the Aleutian Islands (AL). Caudal gluteal venous blood samples were collected into the anticoagulant, ethylenediaminetetraacetic acid (EDTA Vacutainer® CPT™; Becton Dickinson, Franklin, NJ) for isolation of peripheral blood leukocytes (PBLs) (Bowen et al., 2004). Extraction of DNA and RNA from PBLs was performed according to methods in Bowen et al. (2004, 2005).

A primer pair recognizing the flanking regions of the putative peptide-binding site (based upon sequence data from the closely-related California sea lion (CSL; *Zalophus californianus*) DRBURN and DRBlociR) was used to amplify SSL (*Euju*)-*DRB* exon 2 and the preceding intron (Table 1). PCR amplifications using these class II primers were performed on 20 ng of each SSL DNA using protocols described in Bowen et al. (2004). The PCR was performed on an MJ Research PTC-200 thermal cycler (MJ Research, Watertown, MA) and consisted of five cycles at 94°C for 30 seconds, 30 cycles at 60°C for 30 seconds, and 72°C for 2 minutes, ending with an extension step of 72°C for 10 minutes. The products of these reactions were electrophoresed on 1.5% agarose gels and visualized by ethidium bromide staining. Bands representing PCR products of the predicted size were excised from the gel, extracted, purified, cloned, and sequenced (Bowen et al., 2004). Nucleotide sequences of the amplicons were analyzed using Align™ and Contig™ sequence alignment software programs (Vector NTI™, Informax Inc, North Bethesda, MD). Based upon these sequences, a new forward primer, located in the intron preceding exon 2, was designed (DRBDNAintron) (Table 1). Subsequent PCR reactions were performed using the primer pair (DRBDNAintron and DRB lociR) on SSL DNA using the conditions described above. Cloning and sequencing were performed to characterize *Euju-DRB* loci.

Table 1. Primer Pairs for Quantification of Steller Sea Lion MHC DRB

Name	Primer sequence
ZCDRBURN	CCCTCCCTTGGCTTGGGCTAG
ZCDRBLEX23	GTAGGCTCAACTCGCCGCTGC
DRBlociR	CTCGCCGCTGCRCCRKGAAG
DRBDNAintron	GGATSSTTCGTGTCCCCACAG
ZCDRB194A	TCATTTCTTGGAGCTGTTGAAGGG
ZCDRB194B	TCATTTCTTGCTCCTGTTTAAGG
ZCDRB194C	TCATTTCTTGGAGCTGTTGAAGGC
ZCDRB194D	TCATTTCTTGCTCCTGTGTAAGTC
ZCDRB194E	TCATTTCTTGCACCTGTGTAAGGC
ZCDRB194F	TCATTTCTTGCACCTGTTTAAGGC
ZCDRB194G	TCATTTCTTGGAGCTGTTTAAGGC
ZCDRB194H	TCATTTCTTGCTCCTGTGTAAGGC
ZCDRB194J	TCATTTCTTGCTCCTGTGTAAGGG
EujuS9F	AGATGAAGCTGGATTACATCCTGGG
EujuS9R	CTTCCTCTTCACACGGCCTGGGC

Reverse transcription was performed on RNA samples from six Steller sea lions according to protocols in Bowen et al. (2004). Based upon data gained from cloning and sequencing *Euju-DRB*, we were able to use eight sequence-specific primer (SSP) pairs previously designed for CSL and one newly identified SSP *Euju-DRB.J* in combination with DRBlociR (Table 1) (Bowen et al., 2004). Genomic DNA from each animal was analyzed with all nine SSPs using an intercalating fluorescent dye PCR (Bowen et al., 2004). Amplifications were performed in an iCycler (BioRad, Hercules, CA) under the following conditions: 2 minutes at 50°C, followed by 15 minutes at 95°C, and 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, and

Table 2. Relative Expression Levels of *Euju-DRB.A–Euju-DRB.J* in Six Steller Sea Lions from Southeast Alaska, Prince William Sound, and the Aleutian Islands[a]

	Age (months)	Sex	A	B	C	D	E	F	G	H	J
SSL 056 SE	2	F	89,579	0	1575	1	177	109	7	408	0
SSL 140 SE	14	F	113,477	0	3112	3	306	95	9	497	0
SSL 065 SE	7	M	212,927	10,086	28,399	3	11,900	49,778	16	1890	0
SSL 090 PWS	23	F	152	1	131,387	2	482	249	210,719	672	0
SSL 302 AL	9	M	0	27,645	49,667	0	36,419	99,334	198,668	3216	0
SSL 263 AL	15	M	102	7149	67,888	3	778	286	136,428	5051	57,926

SSL, Steller sea lions; SE, Southeast Alaska; PWS, Prince William Sound; AL, Aleutian Islands.
[a]Determined by $2^{-\Delta\Delta CT}$ method.

72°C for 30 seconds, with a final extension step of 72°C for 10 minutes. Reaction specificity was monitored by melting curve analysis using a final data acquisition phase of 60 cycles of 65°C for 30 seconds and verified by direct sequencing of randomly selected amplicons (Bowen et al., 2004).

cDNA was examined with all nine SSL SSPs and a control gene, ribosomal subunit S9 SSP (Table 1). Quantitative PCR systems for SSL S9 and MHC genes were conducted in individual wells. Each reaction contained 500 ng DNA in 25 µl volumes with 20 pmol SSP, 20 pmol DRBLex23, Tris-Cl, KCl, $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$ (pH 8.7), dNTPs, HotStar Taq DNA Polymerase (Quantitect SYBR Green PCR Master Mix, Qiagen, Valencia, CA), and 0.5 units uracil-N-glycosylase (Roche, Indianapolis, IN). Amplifications were performed in an iCycler (BioRad) under the following conditions; 2 minutes at 50°C, followed by 15 minutes at 95°C, and 35 cycles at 94°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, with a final extension step of 72°C for 10 minutes. Reaction specificity was monitored by melting curve analysis using a final data acquisition phase of 60 cycles at 65°C for 30 seconds and verified by direct sequencing of randomly selected amplicons. Additionally, expression levels of all nine SSL *DRB* gene sequences were established from six Steller sea lions.

RNA preparations, with and without prior DNase treatment and with or without RT-step, were subjected to quantitative PCR to confirm the absence of contaminating gDNA. None of the PCR systems produced a signal when DNase treated RT^- RNA samples were used for the amplification.

PCR products of the target genes and S9 were randomly chosen for sequencing to verify analytical specificity using standard sequencing procedures (ABI 377 DNA sequencer).

Data were expressed relative to a reference sample, called the calibrator (weakest signal), for relative quantitation by the comparative C_T method. The C_T for the target amplicon and the C_T for the endogenous control were determined for each sample. Differences in the C_T for the target and the C_T for the endogenous control (ΔC_T) were calculated to normalize for differences in RNA extractions and efficiency of the RT step. The ΔC_T for each experimental sample was subtracted from the ΔC_T of the calibrator resulting in a $\Delta\Delta C_T$ value. Lastly, the amount of target, normalized to the endogenous control and relative to the calibrator, was calculated by $2^{-\Delta\Delta CT}$. Thus, all experimental samples are expressed as an *n*-fold difference relative to the calibrator (Leutenegger et al., 1999).

Examination of genomic DNA from 10 SSLs revealed the presence of nine MHC DRB sequence patterns, all being present in each individual. The lack of sequence configuration differences among these 10 individuals is in contrast to the wide range of MHC *DRB* sequence configurations recently described within populations and rookeries of CSLs (Bowen et al., 2004, 2005).

Identification of real-time pressure on the SSL immune system, not yet reflected at the genomic level, was identified by quantitation of mRNA encoded by each individual MHC *DRB* gene. Extensive variation in the expression of MHC DRB genes was identified (Table 2). Although present in the genomic DNA, select *Euju-DRB* genes were either relatively reduced in expression or completely absent (Table 2). Only one animal (AL) expressed all nine *Euju-DRB* genes. *Euju-DRB.A* and *.G* were both differentially expressed with three animals expressing relatively less of *DRB.A* and the other three expressing relatively less of

DRB.G. *Euju-DRB.*B was not expressed in two of the three SE animals, *DRB.D* was not expressed in one of the AL animals, and *DRB.J* was not expressed in five of the six animals.

Retrospective studies have suggested that population bottlenecks, resulting in loss of genetic diversity within the MHC gene complex, have precipitated an increased susceptibility to emerging infectious diseases (Yuhki and O'Brien, 1990; Hedrick, 1994; Hughes and Hughes, 1995). The current study presents a unique approach to complement classic genotyping data by providing real-time assessment of perturbations in these functionally relevant MHC genes. Expression of MHC class II genes is highly regulated at the level of gene transcription by multiple factors. While limited studies have suggested that control of select MHC genes can be differentially regulated through promoter polymorphism (Andersen et al., 1991; Berggren and Seddon, 2005), extensive literature has established relationships between physiologic insults and reduced MHC class II expression (Snyder and Unanue, 1982; Schwab et al., 2005). The rationale for our approach was supported by the demonstration that environmental contaminants/chemicals and nutritional stress can result in suppression of the expression (gene transcription) of select MHC genes (Hughes et al., 1996; Dong et al., 1997; Venkatraman and Pendergast, 2002). The utility of this approach is in its ability to provide a more timely indicator of changes in ecosystem health.

While the numbers of animals examined in this study are extremely limited, the demonstration of differential MHC gene expression in animals from geographically disparate regions is intriguing. The Southeast Alaska population has been increasing for a long period of time, perhaps the past 90 years or so. The Prince William Sound subpopulation has been relatively stable since about 1975, while the Aleutian subpopulation has declined by perhaps 80%–90% since about 1975 (Trites and Larkin, 1996).

These preliminary findings appear to suggest MHC gene expression in marine mammals might be useful as an early warning indicator of environmental stress and ecosystem degradation (Tabor and Aguirre, 2004). However, to demonstrate that the variation in MHC is meaningful in this regard, further research is needed to establish statistically consistent patterns within static populations, and differences between populations within unique environments. This would be most effectively realized through the temporal analysis of captive sea lions living in controlled environments.

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